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Title: Cyanobacteria and eukaryotic algae use different chemical variants of vitamin B_{12}

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SUMMARY

Eukaryotic microalgae and prokaryotic cyanobacteria are the major components of the phytoplankton. Determining factors that govern growth of these primary producers, and how they interact, is therefore essential to understanding aquatic ecosystem productivity. Over half of microalgal species representing marine and freshwater habitats require for growth the corrinoid cofactor B_{12}, which is synthesised *de novo* only by certain prokaryotes, including the majority of cyanobacteria. There are several chemical variants of B_{12}, which are not necessarily functionally interchangeable. Cobalamin, the form bioavailable to humans, has as its lower axial ligand 5,6-dimethylbenzimidazole (DMB). Here we show that the abundant marine cyanobacterium *Synechococcus* synthesises only pseudocobalamin, in which the lower axial ligand is adenine. Moreover bioinformatic searches of over 100 sequenced cyanobacterial genomes for B_{12} biosynthesis genes, including those involved in nucleotide loop assembly, suggests this is the form synthesised by cyanobacteria more broadly. We further demonstrate that pseudocobalamin is several orders of magnitude less bioavailable than cobalamin to several B_{12}-dependent microalgae representing diverse lineages. This indicates that the two major phytoplankton groups use a different B_{12} currency. However, in an intriguing twist, some microalgal species can use pseudocobalamin if DMB is provided, suggesting that they are able to remodel the cofactor, whereas *Synechococcus* cannot. This species-specific attribute implicates algal remodellers as novel and keystone players of the B_{12} cycle, transforming our perception of the dynamics and complexity of the flux of this nutrient in aquatic ecosystems.

INTRODUCTION

Eukaryotic microalgae are photosynthetic microbes estimated to be responsible for ~50% of global carbon fixation [1]. Elucidation of factors that control algal community structure and dynamics is thus fundamental to understanding the global cycling of carbon. Nutrients such as iron, nitrogen and phosphorus clearly play an important role [2], but many microalgae also require the vitamins B_1 (thiamine), B_7 (biotin) or B_{12} for growth [3]. B_{12} is required as a cofactor for methionine synthase (METH) activity, a key enzyme of cellular one-carbon (C1) metabolism important for production of the universal methyl donor S-adenosylmethionine (SAM), and for folate cycling necessary for DNA synthesis [4]. Those algae that do not need a supply of B_{12} cannot synthesise the vitamin, rather they possess an alternative form of methionine synthase (METE) that can catalyse the same reaction in a B_{12}-independent fashion [5, 6].

Measurement of B_{12} levels in the water column have indicated concentrations of ~10 pM in freshwater ecosystems [7], and are often below the threshold of detection in certain marine habitats, including large areas of the northeast Pacific margin [8]. The scarcity of this micronutrient is therefore thought to limit phytoplankton abundance [8], so competition for B_{12} amongst those organisms that require/use it is likely. Indeed field enrichment experiments found that whereas N-addition stimulated all microbial growth, there was a specific growth enhancement of phytoplankton >5 µm (i.e. the larger eukaryote fraction) with B_{12} supplementation [9]. However, several recent studies have demonstrated that heterotrophic bacteria can satisfy microalgal requirements for B_{12} via mutualistic interactions [e.g. 5, 10].

Provision from prokaryotes is particularly pertinent since the biosynthetic pathway of this cofactor is confined to certain archaea and bacteria. B_{12} is an umbrella term that refers to cobalt-containing corrinoids (ring-contracted tetrapyrroles), which have upper and lower axial ligands to the cobalt ion (Figure 1A). The nature of these ligands varies, leading to diversity in the structural forms of B_{12}. Methylcobalamin, where the upper axial ligand is a methyl group, is involved in methyl-transfer reactions, whereas adenosylcobalamin (coenzyme B_{12}) is used for radical based rearrangements and reductions [11]. The identity of the base found as the lower axial ligand, bound via a nucleotide loop, may vary too. In cobalamin, the base is 5, 6-dimethylbenzimidazole (DMB).

Many bacteria, including methanogens and anaerobes such as *Clostridium* species, synthesise a B_{12} vitamer known as pseudocobalamin [12, 13], which differs from cobalamin in that DMB is replaced by adenine. Intrinsic factor, the mammalian B_{12}-binding protein important in uptake from the gut, has a lower affinity for pseudocobalamin than cobalamin [14]. Pseudocobalamin is therefore considered not 'bioavailable' to humans, and the efficacy of vitamin supplements produced from cyanobacteria such as *Spirulina* and *Aphanizomenon*, which also contain pseudocobalamin, has been questioned [15, 16].

Cyanobacteria are the numerically dominant photosynthetic microbes in the marine environment [17]. Two cyanobacterial species, *Crocosphaera watsonii* WH8501 and *Synechococcus* sp. WH7803, were reported to release B_{12} into the media at rates exceeding those estimated for the heterotrophic bacterium *Halomonas*, suggesting that cyanobacteria might be the major source of B_{12} for marine algae [18]. However, indications from the early literature suggest consideration of algal specificity towards different B_{12}-like factors may be pertinent [19]. Here we investigate corrinoids in several strains of *Synechococcus*, an abundant and ubiquitous marine cyanobacterium [17, 20], the nature of their axial ligands, and their ability to support growth of eukaryotic primary producers.

RESULTS

Synechococcus species make only pseudocobalamin

The biosynthesis of the corrinoid ring of B_{12} from the common tetrapyrrole progenitor uroporphyrinogen III requires at least 20 enzymatic steps, and several routes are known [21]. In a preliminary investigation, Sañudo-Wilhelmy *et al*. (2014) [22] searched for the presence of B_{12}-biosynthesis genes in ~40 marine cyanobacteria with sequenced genomes. They found that all but one species had putative homologues for at least 11 of these genes, and so concluded that they were capable of making B_{12}. However, they did not investigate the genes involved in synthesis of the lower axial ligand and so could not conclude whether the cyanobacteria studied produced cobalamin or pseudocobalamin. To refine the analysis of B_{12}-biosynthesis genes we searched 123 sequenced cyanobacterial genomes for all 20 genes of the corrinoid pathway. All but six species contained at least 15/20 of these genes and were predicted to be B_{12} producers (Dataset S1; Supplemental

105 Experimental Procedures). Additionally, we searched for genes involved in DMB biosynthesis, for
106 which two routes are currently known [23, 24]. The BluB enzyme, first characterised in
107 *Sinorhizobium meliloti* (Rhizobia) makes DMB from riboflavin under aerobic conditions [23]. Using
108 this sequence as a query, no hits were found in 118 cyanobacterial genomes including *C. watsonii*
109 WH8501 and *Synechococcus* sp. WH7803. For 5 species (including 3 from the *Fischerella* genus),
110 hits for BluB were obtained: two were annotated as cob(II)yrinic acid *a,c*-diamide reductase (CobR),
111 which is an enzyme of an earlier stage of B_{12} biosynthesis, whereas the others were unknown. In
112 contrast, BluB homologues were found in 80% of sequenced rhizobia [227/284 genomes, Dataset
113 S1C] and 60% of Rhodobacterales species [77/128, Dataset S1D) including *Mesorhizobium loti*,
114 *Sinorhizobium meliloti* and *Rhizobium leguminosarum* and the marine bacterium *Dinoroseobacter*
115 *shibae*, all of which can support algal B_{12}-auxotrophic growth [10, 25]. More recently a second route
116 for DMB biosynthesis was identified in the obligate anaerobic bacterium *Eubacterium limosum* [24],
117 and enzymes encoded by the *bzaABCDE* operon were shown to direct DMB production via an
118 oxygen-sensitive reaction from the purine precursor 5-aminoimidazole ribotide (AIR) [24]. We found
119 that none of the cyanobacterial genomes encoded the full *bzaABCDE* operon (Dataset S1B).
120 Moreover, CobT, which is required for DMB activation, is absent from all but two cyanobacterial
121 genomes. Taken together these searches suggest that the vast majority of cyanobacteria cannot make
122 DMB, and hence cannot make cobalamin either.

123 To validate the observations from the bioinformatics analysis, we wanted to assess directly
124 what corrinoids are synthesized by cyanobacteria, and investigated the B_{12} content of strains of marine
125 *Synechococcus*, since this is an ancient and ecologically abundant lineage [20], with a mean global
126 abundance of $7.0 \pm 0.3 \times 10^{26}$ cells y^{-1} and high biomass-specific CO_2 fixation rates [17, 26], and
127 axenic strains are available. Corrinoids can be extracted from cells as their cyano-derivatives and then
128 analysed by HPLC-mass spectrometry (LC-MS). First, using purified cyanocobalamin (obtained
129 commercially) and cyanopseudocobalamin, prepared from *Propionibacterium acidi-propionici* [12]
130 we were able to distinguish the two variants of B_{12} by their different retention times on the LC (Figure
131 1B) and different mass (Figure S1A and B). Derivatised cell lysate obtained from axenic cultures of
132 the heterotrophic marine bacterium *D. shibae* DFL12T contained only cyanocobalamin. We next

tested five members of the *Synechococcus* lineage representing different clades and habitats (highlighted in red in Figure S2): coastal strain CC9311 (sub-cluster (SC) 5.1A, Clade I), oligotroph WH8102 (SC5.1A, Clade III), WH7803 and WH7805 (SC5.1B, Clades V and VI respectively) which are widely distributed in various oceanic waters, and the estuarine strain WH5701 (SC5.2). A single peak was observable in these samples at a retention time consistent with the pseudocobalamin standard (Figure 1B), and its identity was confirmed by MS (Figure S1D-H). To facilitate subsequent physiological work, we also tested two model freshwater cyanobacterial species *Synechocystis* sp. PCC6803 and *Synechococcus* e*longatus* PCC7942, since these species grow quickly and easily in the laboratory. Again, cell lysates from these strains contained only pseudocobalamin (Figure 1B; Figure S1I and J). Together these data demonstrate that the cyanobacterial species sampled here make only pseudocobalamin in axenic laboratory culture conditions.

However, some B_{12}-synthesising bacteria can modify endogenous B_{12} forms with an alternative base [27]. For instance although *Salmonella enterica* cannot make DMB, it can import it and then make cobalamin instead of pseudocobalamin [27]. To investigate whether *Synechococcus* can perform this so-called 'guided biosynthesis', we grew strains WH8102 and WH7803 (representing SC5.1A and 5.1B) in the presence of pseudocobalamin and 1 µM DMB, but only pseudocobalamin was detected (Figure1B; Figure S1K and L). We conclude therefore that *Synechococcus* cannot replace the adenine base with DMB to make cobalamin.

Pseudocobalamin is orders of magnitude less bioavailable to eukaryotic algae

We next tested whether cyanobacterially derived B_{12} could be utilised by eukaryotic algae. Cell-free extracts of *S. elongatus* PCC7942 (pseudocobalamin producer) were unable to rescue growth of the B_{12}-dependent freshwater alga *Lobomonas rostrata*, whereas there is clear growth with the addition of extracts of 3 rhizobial bacteria (Figure S3), which all encode BluB [28]. It is conceivable that the growth is due to other compounds in the crude lysate, so this initial experiment was extended using the purified compounds, cyanocobalamin and cyanopseudocobalamin. Equivalent concentrations of each B_{12} variant were added to axenic cultures of B_{12}-dependent microalgae from different algal lineages: marine species *Ostreococcus tauri* (Chlorophyta, Mamiellophyceae), *Amphidinium carterae*

(Alveolata, Dinoflagellate), *Pavlova lutheri* (Haptophyta, Prymnesiophyceae), *Thalassiosira pseudonana* (Heterokontophyta, Coscinodiscophyceae), *Aureococcus anophagefferens* (Heterokontophyta, Pelagophyceae), and the freshwater species *Euglena gracilis* (Excavata, Euglenozoa) and *L. rostrata* (Chlorophyta, Volvocales). We also tested a B_{12}-dependent *metE* mutant of *Chlamydomonas reinhardtii* (Chlorophyta, Volvocales) [29]. When pseudocobalamin was supplied at a concentration of 0.07 or 0.7 nM we observed little or no growth in any of the marine species, nor with the *C. reinhardtii metE* mutant or *L. rostrata.* This is in contrast to cobalamin, which supported growth of all algal cultures at equivalent concentrations (Figure 2) (Student's t-test, $P<0.05$, n=3). For *O. tauri, A. carterae*, and *T. pseudonana,* and to a lesser extent the *C. reinhardtii metE* mutant, provision of pseudocobalamin at 7 nM (~10µg/L) supported growth to a similar extent as cobalamin (Figure 2A, B, D, H), although this amount is significantly higher than found in natural ecosystems (with reported concentrations ranging from below the detection threshold to 0.03 nM large areas of the northeast Pacific margin, for instance) [7, 8]. One way to compare the efficacy of the different B_{12} variants is to carry out dose-response experiments, which enable determination of an EC_{50} (that is the concentration required to support half-maximal biomass accumulation) and also provide an indication of the minimum amount needed to observe any growth, and so we carried these out with the *C. reinhardtii metE* mutant. Figure S4 shows that the EC_{50} was ~0.07 nM for cobalamin, compared to ~7 nM (~100-fold higher) for pseudocobalamin. In addition, it is clear that even the highest concentration of pseudocobalamin used (40 nM) is not saturating, whereas 0.2 nM cobalamin supports maximum growth. For *E. gracilis* some growth was observed even at the lowest pseudocobalamin concentration, but it was still significantly lower than with cobalamin (Figure 2G). Thus, pseudocobalamin is orders of magnitude less bioavailable to eukaryotic algae. It is notable to mention that *E. gracilis* has also been demonstrated to encode a B_{12}-dependent (type II) ribonucleotide reductase [30], which could account for the growth response to pseudocobalamin observed in this alga.

We reasoned that the reduced ability of pseudocobalamin to support growth of algal B_{12}-auxotrophs may be either because the molecule cannot be used as a cofactor, or because it does not get transported into algal cells. To investigate the latter possibility, we took advantage of the presence of B_{12}-responsive genes previously identified in the marine diatom *Phaeodactylum tricornutum* and *C.*

reinhardtii [31, 32]. These algae do not need B_{12} for growth, but will uptake and use it if it is available [4, 6]. Several genes in these algae are responsive to B_{12}: *METE* (in *P. tricornutum* and *C. reinhardtii* [6, 31, 32]), *CBA1*, encoding a novel cobalamin acquisition protein (in *P. tricornutum* only [31]), and S-adenosylhomocysteine hydrolase, *SAHH* (in *C. reinhardtii* only [32]). Using RT-qPCR we analysed their expression in cells grown in the presence of 0.7 nM cobalamin or pseudocobalamin. For *P. tricornutum* both forms of B_{12} resulted in down-regulation of *METE*, but the effect was less pronounced with pseudocobalamin compared to that with cobalamin (Student's t-test, $P<0.001$, n=3) (Figure 3A). As previously demonstrated, cobalamin supressed *CBA1* [31] but this gene was significantly up-regulated by pseudocobalamin (Student's t-test, $P<0.05$, n=3) (Figure 3A). In *C. reinhardtii* both *METE* (Student's t-test, $P<0.05$, n=3) and *SAHH* (Student's t-test, $P<0.01$, n=3) were down-regulated relative to the no supplementation control (Figure 3B) with both forms of B_{12}. Subsequent western blot analysis using polyclonal antibodies against *C. reinhardtii* METE protein [32] demonstrated a modest reduction of METE abundance when cells were grown with pseudocobalamin, although not to the same extent as cobalamin (Figure S5). Nonetheless, the effect of pseudocobalamin on the expression of these four B_{12}-responsive genes indicates that the molecule can enter both *C. reinhardtii* and *P. tricornutum* cells.

Certain algae are capable of remodelling pseudocobalamin

Some bacteria that do not make B_{12} can modify imported forms with an alternative base, via 'corrinoid re-modelling' [33, 34]. To investigate this possibility in algae, we grew B_{12}-requiring species in the presence of pseudocobalamin and a range of DMB concentrations. For most, growth was not restored by DMB supplementation (Figure 4A, B, D-G). However, for *P. lutheri*, and the *C. reinhardtii metE* mutant, addition of DMB alongside pseudocobalamin rescued growth to the same extent as cobalamin (Figure 4C and H). A dose-response experiment with *P. lutheri* established an EC_{50} value of ~18 pM for cobalamin (Figure 5A). A similar experiment with a fixed concentration (0.7 nM) of pseudocobalamin but varying the amount of DMB revealed a similar EC_{50} (~23 pM) (Figure 5B). Interestingly, an equivalent dose response curve (and EC_{50} value: ~26 pM) was observed when cells were grown in medium made using natural filtered seawater rather than artificial sea salts. Thus the level of DMB in the natural filtered seawater is not sufficient to allow remodelling,

otherwise the dose-response curve would be shifted to the left. That comparable levels of B_{12} and DMB (at a fixed level of pseudocobalamin) are able to rescue B_{12}-dependent growth implies that *P. lutheri* is remodelling pseudocobalamin with DMB to generate cobalamin. Dosage experiments with the *C. reinhardtii* B_{12}-dependent *metE* mutant also identified similar EC_{50} values of ~28 pM and ~70 pM for cobalamin and DMB respectively (Figure 5C and D). In this case the EC_{50} value for DMB was slightly higher than cobalamin. We also tested whether *C. reinhardtii* is capable of *de novo* lower-loop synthesis, and grew the *C. reinhardtii* B_{12}-dependent *metE* mutant with DMB, alongside (dicyano)cobinamide, a B_{12} precursor that lacks the DMB ribonucleotide tail. In this case we saw no restoration of growth (Figure 6A).

Our data indicate that of eight diverse algal species studied, six do not appear to be able to use exogenous DMB. Nevertheless the observation that growth of the *C. reinhardtii* B_{12}-dependent *metE* mutant (alongside that of *P. lutheri*) with pseudocobalamin is restored by DMB provision suggests that these algae are able to chemically modify pseudocobalamin to a form that can support B_{12}-auxotrophic growth. To test more directly whether pseudocobalamin is being remodelled, we grew samples of *C. reinhardtii* in the presence of i) cobalamin, ii) pseudocobalamin (1 nM) and iii) pseudocobalamin (1 nM) +DMB (1 µM) and prepared cell lysate for LC-MS analysis. However, we could not detect any form of B_{12} from lysed cells. We infer from this that intracellular B_{12} levels are extremely low i.e. the quantity from ~1×10^{10} cells is below the threshold detection of the LC-MS (which in our system is ~1×10^{12} molecules). Without a clear idea of what order of magnitude more biomass would be required, and constrained by the limitations of scale we turned to alternative means of characterising remodelling activity and investigated the effect of DMB + pseudocobalamin on gene expression in *C. reinhardtii*. Previously, we had generated several transgenic lines of *C. reinhardtii* expressing the B_{12}-responsive element of the *METE* gene fused to the *BLE* gene, which confers resistance to the antibiotic zeocin [32]. This reporter gene construct enables rapid and easy measurement of B_{12}-responsive gene expression, whereby growth with cobalamin represses expression of *BLE* so that cells die in the presence of zeocin (Figure 6B). In contrast pseudocobalamin alone had little effect, but the inclusion of DMB impaired growth to the same extent as cobalamin,

244 demonstrating that *C. reinhardtii* converts DMB and pseudocobalamin into a form that is able to
245 repress the *METE* promoter.

246 The pathway for pseudocobalamin re-modelling has been investigated previously in the
247 purple bacterium *Rhodobacter sphaeroides*, and cobinamide amidohydrolase (CbiZ) and cobinamide-
248 phosphate synthase (CbiB) have been implicated in this process [34]. We could not identify *cbiZ*, or
249 *cbiB* in any of the algal genomes we analysed. We therefore searched for proteins shown to be
250 involved in lower-loop assembly and activation [34] in *S. enterica* (CobT, CobS and CobC) [27],
251 where mutants of CobT are unable to incorporate exogenous DMB (Figure 6C). We identified genes
252 encoding all three of these proteins in *C. reinhardtii*, which exhibits the remodelling phenotype (Table
253 S1; Figure 5). In contrast, BLASTP searches of the genomes of *O. tauri* and *T. pseudonana*, species
254 that do not appear to remodel, were negative for CobT and CobS. Although a hit for CobC was
255 identified in *T. pseudonana*, it should be noted that CobC catalyzes the dephosphorylation of the
256 AdoCbi-GDP precursor and therefore BLAST searches may retrieve genes encoding unrelated
257 kinases. Interestingly, we identified hits for CobT and CobC but not CobS in *A. anophagefferens*,
258 which can use pseudocobalamin but only with very high levels of DMB (10 µM) (Figure 4E). A
259 transcriptome sequence for *P. lutheri* is available via the Marine Microbial Eukaryote Transcriptome
260 Sequencing Project (MMETSP), a transcriptome database of 396 unique strains representing
261 ecologically significant and taxonomically diverse marine microbial eukaryotes [35]. This alga, which
262 can use pseudocobalamin alongside DMB, also expresses *COBT*, *COBS* and *COBC* (Dataset S2A).
263 Thus the presence of these novel proteins correlates with the ability to remodel pseudocobalamin,
264 implicating them in B_{12} metabolism. We also identified another 46 candidate remodellers in the
265 MMETSP (Dataset S2A), including several that encoded *METE*, and so are like *C. reinhardtii* in
266 being independent of a source of B_{12} for growth. In total the potential remodellers represented ~11%
267 of unique strains, and included representatives of the higher class levels Alveolata, Stramenopila,
268 Hacrobia, and Viridiplantae (Dataset S2B). Incidentally, none of the
269 sequenced *Synechococcus* genomes encode CobT (Dataset S1), which might explain
270 why *Synechococcus* strains cannot remodel pseudocobalamin to cobalamin in the presence of DMB
271 (Figure 1B).

DISCUSSION

Eukaryotic microalgae and cyanobacteria are the major components of the phytoplankton in marine and freshwater systems. Since they both inhabit the photic zone, they will compete for resources including light and limiting nutrients such as nitrogen and Fe. We have demonstrated that, in contrast to heterotrophic bacteria such as *D. shibae* (and certain rhizobial bacteria [28]) that make cobalamin, members of the ubiquitous marine *Synechococcus* genus synthesise only pseudocobalamin, in which the lower base is adenine instead of DMB (Figure 1). Moreover, a survey of diverse cyanobacterial genomes, encompassing marine and freshwater species, showed the vast majority lack BluB and the *bzaABCDE* operon (Dataset S1) [23, 24]. This strongly suggests that pseudocobalamin is the major form of B_{12} synthesised by most if not all cyanobacteria.

We found that pseudocobalamin is considerably less bioavailable than cobalamin to several B_{12}-dependent algae (Figure 2). This reduced bioavailability suggests these organisms are compromised in their ability to acquire or use pseudocobalamin as a cofactor. Human intrinsic factor, part of the B_{12} uptake system in the gut, exhibits a 500-fold-lower binding affinity for pseudocobalamin [14], thus reducing the bioavailability of the compound to humans. In algae, currently only one protein has been implicated in B_{12} uptake [31] (CBA1), although the precise molecular mechanism and role of CBA1 in B_{12} binding are not fully understood. Nevertheless, the ability of pseudocobalamin to affect the expression of algal B_{12}-responsive genes (Figure 3) and protein levels (Figure S5) suggests this compound can enter algal cells, albeit that it has the opposite effect on *CBA1* to cobalamin, suggesting that the cells are experiencing cobalamin-deficiency. Transport of pseudocobalamin into the cell is also indicated by our observed remodelling of pseudocobalamin in *C. reinhardtii* and *P. lutheri* following DMB addition (Figure 4 and 5). The identification of genes encoding enzymes of lower ligand activation (COBT) and nucleotide-loop assembly (COBS) [27] in these algae (Table S1) provides a likely mechanism for corrinoid remodelling. We found no evidence of secretory peptide signals in *C. reinhardtii* COBT or COBS using the green algal subcellular localization tool 'PredAlgo' [36], indicating that remodelling presumably takes place within the cell, and providing further support for the ability of

pseudocobalamin to be taken up. Whether these genes have been acquired through lateral gene transfer from a bacterial source, which is thought to be the case for *E.* coli [37], remains unknown. However, of the non-algal sequences retrieved via a BLAST search of the NCBI non-redundant database with the *C. reinhardtii COBT/COBS* top hits were derived from the amphipod associated protist species *Aphanomyces astaci* ($4e^{-57}$) and *Sphaeroforma arctica* ($7e^{-77}$), respectively. A broader phylogenetic analysis of *COB* genes will be integral to further understanding of what, at a first glance, appears to be an intriguing evolutionary history. Since algae rely on B_{12} for METH [5], the function of pseudocobalamin as a cofactor for this enzyme is also an important question. Structural data available for the B_{12}-binding pocket and the active site of METH [38, 39] implicates several amino acids in B_{12} binding, with the DMB 'tail' buried within a cleft of the active site [40]. Since pseudocobalamin contains an alternative lower base to B_{12} it seems plausible that algal METH proteins may have reduced binding affinity for pseudocobalamin.

The combination of DMB with pseudocobalamin improves the bioavailability to certain algae. We infer from this that these remodelling algae are able to generate cobalamin from pseudocobalamin + DMB, although we were unable to measure detectable levels of any form of B_{12} in *C. reinhardtii* cells grown with pseudocobalamin plus DMB. It is possible therefore that another form of the vitamin is being generated, though we deem this unlikely. In any case, our results highlight the importance of considering environmental concentrations of DMB. A bioassay to measure free DMB concentrations was recently reported [41]. Analysis of samples derived from host-associated (termite/rumen) and natural environmental samples (*Eucalyptus* grove soil/creek water) determined concentrations in the picomolar and sub-picomolar range. Our observation that *P. lutheri* (whose growth can be supported by pseudocobalamin + DMB) could not grow in natural seawater from the English Channel supplemented with pseudocobalamin (Figure 5B), suggests DMB levels were not sufficient in these coastal waters to support remodelling. Nevertheless, further work is required to quantify DMB in marine (and freshwater) environments more broadly. In a similar vein, whilst a recent field study by Heal *et al* (2015) quantified the relative abundance of four upper axial variants of

B_{12} (CN, Me, Ado, and OH) in estuarine waters of the Puget Sound, levels of lower axial ligand variants are unknown [42].

Members of all three domains of life require B_{12}, yet its biosynthesis is limited to a subset of prokaryotes. As such, the flux of B_{12} between microbes is integral to the growth of auxotrophic species. Our results imply heterotrophic bacteria are likely to be a more important source of B_{12} for eukaryotic algae than cyanobacteria. An increasing body of evidence, provided by this study and others [33, 34, 43, 44], suggests the relationship between requirers and providers has become blurred by the existence of scavengers, and re-modellers. That different B_{12} forms are not functionally equivalent between organisms means that biochemical transformations between vitamer classes are essential for this micronutrient to reach different members of the community. This complicates our current view of B_{12} cycling in aquatic environments (Figure 7). Whether cyanobacteria synthesise a currency of B_{12} that is inaccessible to competing eukaryotic microbes as a strategy to exclude competitors remains unknown. Nevertheless, the observation that certain algae possess a counter-mechanism, to convert pseudocobalamin to a bioavailable form, suggests the selective pressure to devise and refine strategies of B_{12} acquisition/utilization in order to elevate accessibility to this limiting micronutrient is strong. In any case, the importance of B_{12} and its derivatives in structuring microbial communities in aquatic ecosystems may have been previously underestimated.

EXPERIMENTAL PROCEDURES

Bioinformatics approaches

A full description of sequence similarity search parameters is provided in the Supplemental Experimental Procedures.

Chemicals

Upper axial cyano forms of cobalamin/pseudocobalamin were used for all B_{12}-amendment experiments. Cyanocobalamin was purchased from Sigma-Aldrich UK. Cyanopseudocobalamin was

prepared by guided biosynthesis from a culture of *Propionibacterium acidi-propionici* DSM 20273 as described previously, and confirmed by UV-Vis, CD, mass, and NMR spectroscopic analysis [12].

Strains and growth conditions

Details of microbial strains and culture conditions are provided in the Supplemental Experimental Procedures and Table S2.

Molecular Methods

RNA extraction and reverse transcription quantitative real-time PCR (RT-qPCR)

Total RNA was extracted [6] and treated with the Ambion Turbo DNase-Free Kit to remove genomic DNA. RNA was reverse transcribed into cDNA with SuperScript II (Invitrogen). Details of RT-qPCR are given in the Supplemental Experimental Procedures and Table S3.

Western blotting

Total protein was extracted, and Western blot experiments performed as described in [32].

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Figure Legends:

Figure 1 *Synechococcus* strains synthesise pseudocobalamin rather than cobalamin. A. Structural Variants of $B_{12.}$ **B.** HPLC-MS extracted ion chromatograms for m/z 1355.5 cyanocobalamin and m/z 1344.5 cyanopseudocobalamin (see also Figure S1). The lower tracks displays the chromatograms for cell-free extracts derived from cultures of cyanobacterial species from (Figure S2). Experiments were carried out in triplicate and one replicate representative of each strain is shown.

Figure 2 Pseudocobalamin poorly supports growth of B_{12}-dependent eukaryotic algae. Growth yield (OD_{730}) of algae in liquid medium supplemented with or without cyanocobalamin or cyanopseudocobalamin (at 0.07, 0.7 or 7 nM) in batch culture after at least two transfers (until the cells had died in the -B_{12} treatment). **A.** *O. tauri*, **B.** *A. carterae*, **C.** *P. lutheri*, **D.** *T. pseudonana*, **E.** *A. anophagefferens*, **F.** *L. rostrata* **G.** *E. gracilis*, **H.** *C. reinhardtii* B_{12}-dependent *metE* mutant [27]. Cbl = cyanocobalamin; PsCbl = cyanopseudocobalamin. *P ≤ 0.05, **P ≤ 0.01, ***P≤0.001 compared with the equivalent concentration of Cbl (two-tailed Student's t-test) (mean ± SEM, *n*=3). See also Figure S3 and S4.

Figure 3 Pseudocobalamin affects expression of B_{12}-responsive genes in *P. tricornutum* and *C. reinhardtii*. RT-qPCR analysis of **A.** *METE* and *CBA1* expression in *P. tricornutum* and **B.** *METE* and

SAHH expression in *C. reinhardtii*, without B_{12} or with 0.7 nM Cbl/PsCbl. Expression was normalized using three house-keeping genes: *Histone H4;* 30S, Ribosomal protein S1, *RPS;* and TATA box-binding protein, *TBP for P. tricornutum* and Receptor of activated protein kinase C 1, *RACK 1;* Actin, *ACT;* Ubiquitin, *UBQ* for *C. reinhardtii*. *P ≤ 0.05, **P ≤ 0.01, ***P≤0.001 compared to the $-B_{12}$ treatment (two-tailed Student's t-test) (mean ± SEM, *n*=3). See also Figure S5.

Figure 4 Provision of lower ligand substrate DMB together with pseudocobalamin can support growth of certain B_{12}-dependent algae. Species were grown in liquid medium (Table S2) without or with 0.7 nM cobalamin (open bars) or 0.7 nM pseudocobalamin (shaded bars) in the presence of different DMB concentrations in batch culture over several transfers, or until the cells had died in the $-B_{12}$ treatment. **A.** *O. tauri* (OTH95), **B.** *A. carteri,* **C.** *P. lutheri,* **D.** *T. pseudonana,* **E.** *A. anophagefferens* , **F.** *L. rostrata* **G.** *E. gracilis*, **H.** *C. reinhardtii,* B_{12}-dependent evolved (*metE*$^-$) mutant line [29]. Optical density (OD_{730}) was used to quantify growth (mean ± SEM, *n*=3).

Figure 5 Certain algae can remodel the lower axial ligand of pseudocobalamin with exogenously supplied DMB. Relative growth yield of **A.** *P. lutheri* cells supplemented with different concentrations of cobalamin after 19 days (values of OD_{730} were normalised as a proportion of growth at 1000 pM B_{12}) or **B.** *P. lutheri* cells supplemented with different concentrations of DMB in the presence of 0.7 nM pseudocobalamin (after 19 days) in artificial seawater or natural filtered seawater (values of OD_{730} normalised to growth at 1000 pM DMB). Equivalent experiments with *C. reinhardtii,* evolved B_{12}-dependent *metE* mutant [29] are displayed in panels **C** & **D** after 96 hours growth (mean ± SEM, *n*=3).

Figure 6A. Relative growth yield (OD_{730}) of *C. reinhardtii* B_{12}-dependent mutant grown with DMB alongside (dicyano)cobinamide, a B_{12} precursor that lacks the DMB ribonucleotide tail. Cells were grown in liquid medium (Table S2) with 0.7 nM cobalamin (open bars) 0.7 nM pseudocobalamin (grey bars), or 0.7 nM dicyano)cobinamide (black bars) in the presence of different DMB

concentrations. **B.** Relative growth yield (OD_{730}) of *C. reinhardtii* reporter line containing a zeocin resistance gene controlled by the *METE* promoter [32] after 13 days in the presence (white bar) or absence (black bar) of DMB (1 µM) and 20 µg/mL zeocin without or with 0.7 nM cobalamin or pseudocobalamin. Values of OD_{730} were normalised as a proportion of growth with no B_{12} (mean ± SEM, *n*=3). **C.** The pathway for the activation of DMB and nucleotide loop assembly in *S. enterica* (adapted from [23]. CobT catalyses the attachment of a phosphoribose moiety derived from nicotinate mononucleotide to form α-ribazole phosphate. CobS and CobC catalyse the attachment of the activated base to the cobamide precursor (GDP-cobinamide).

Figure 7 Complex B_{12} cycling in a 'hypothetical' marine microbial community. Cobalamin produced by heterotrophic bacteria such as *Dinoroseobacter* sp. is directly usable by algal B_{12} auxotrophs representing major marine taxa, whereas cyanobacterially-derived pseudocobalamin is not. However, those algae like *P. lutheri* capable of remodelling can access this essential cofactor if DMB is also present.